New Polyoxygenated Triterpenoids from *Stachyurus himalaicus* var. *himalaicus*

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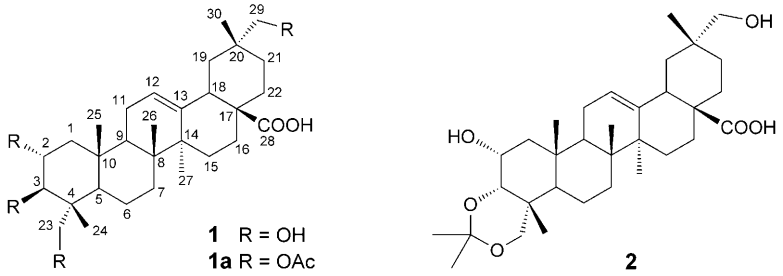
Two new polyoxygenated triterpenoids, stachlic acid A (=(2α,3β)-2,3,23,29-tetrahydroxyolean-12-en-28-oic acid; **1**) and stachlic acid B (=(2α,3α)-2,29-dihydroxy-3,23-[(1,1-dimethylmethylene)dioxy]olean-12-ene-28-oic acid; **2**), were isolated from *Stachyurus himalaicus* var. *himalaicus*. Their structures were established by means of extensive spectroscopic studies and chemical evidence. The purified product **1** was found to have moderate *in vitro* cytotoxic activity against human Hela cells.

Introduction. – Stachyuraceae comprises only the genus *Stachyurus*, which is distributed from the Himalayas to Japan [1]. A literature survey revealed that some tannins have been isolated from the genus before [2][3]. *Stachyurus himalaicus* var. *himalaicus* is a shrub growing at Wenshan County, China. The plant is known as '*tong-cao*' in traditional Chinese medicine (TCM), and has been used as galactopoietic, diuretic, and for the treatment of dropsy and gonorrhea for a long time [1]. However, no work has been reported on the biologically active constituents of this species. A preliminary pharmacological study on this plant showed that its EtOH extract is cytotoxic against human Hela cell lines at a concentration of 10 μg/ml. Further bioassay-guided studies revealed that the AcOEt-soluble fraction of the plant extract displays strong cytotoxic activity.

In the course of our systemic studies on the chemical constituents of *S. himalaicus* var. *himalaicus*, we obtained two new polyoxygenated triterpenoids, stachlic acids A (**1**) and B (**2**), whose isolation and structure elucidation are reported herein.

Results and Discussion. – The twigs and leaves of *S. himalaicus* var. *himalaicus*, collected from Wenshan County, Yunnan Province, were extracted with 95% EtOH. The concentrated extract was suspended in H_2O and successively extracted with petroleum ether (PE), AcOEt, and BuOH. The AcOEt-soluble extract was subjected to column chromatography to yield compounds **1** and **2**, two highly oxygenated triterpenoids with an olean-12-ene skeleton. Their structures were elucidated by detailed spectroscopic analyses and by chemical conversion.

Stachlic acid A (**1**) was obtained as colorless needles. The compound was optically active, with $[\alpha]_D^{25} = 47.7$ ($c = 0.72$, MeOH), and had the molecular formula $C_{30}H_{48}O_6$,



with eleven degrees of unsaturation, as deduced by HR-ESI-MS (*m/z* 527.3341 ($[M+Na]^+$)). The ^{1}H- and ^{13}C-NMR spectra (*Table*), in combination with HMQC, HMBC, and NOESY data (*Fig. 1*), established the structure of **1** as (2*α*,3*β*)-2,3,23,29-tetrahydroxyolean-12-en-28-oic acid, as corroborated by chemical derivatization to the tetraacetate **1a**.

Compound **1** displayed a positive *Liebermann–Burchard* test. The IR spectrum of **1** featured absorptions of OH (3429), C=O (1729), and olefinic (1633 cm^{-1}) groups. Analysis of the ^{13}C-NMR (DEPT) spectrum revealed 30 carbon signals, including five Me, eleven CH_2 (two of them oxygenated), five CH (two of them oxygenated), one trisubstituted C=C bond, and seven quaternary C-atoms including one C=O group (*Table*). The ^{1}H-NMR spectrum of **1** displayed signals at δ(H) 1.05–1.25 due to five Me groups. The downfield *singlet* at δ(H) 5.49 was assigned to a trisubstituted C=C bond. The mass spectrum indicated that, by typical *retro-Diels–Alder* fragmentation of ring *C*, compound **1** produced the protonated fragments *m/z* 265 and 241, which confirmed an olean-12-ene derivative carrying three OH groups at rings *A*/*B*, with two of its Me groups at tertiary C-atoms at rings *D*/*E* being transformed into a COOH and a CH_2OH group, respectively.

The ^{1}H-NMR spectrum of **1** showed a supplementary two-proton *singlet* at δ(H) 3.56, which correlated with a CH_2OH group at δ(C) 74.3 (*t*) in an HMQC experiment. The observation of HMBC cross-peaks between this H-atom and four C-atoms at δ(C) 20.2 (*q*), 29.5 (*t*), 37.0 (*s*), and 41.7 (*t*) suggested that C(29) or C(30) was oxygenated *(Fig. 1)*. As reported in the literature, the ^{1}H-NMR spectrum of an oleanane displays

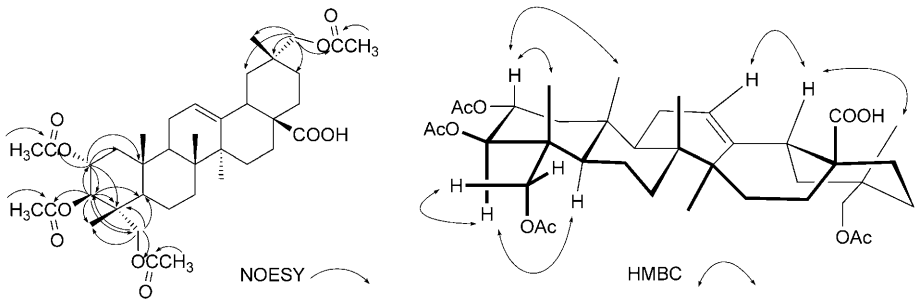


Fig. 1. *Key HMBC and NOESY correlations of* **1a**

Table. *^{13}C-NMR Data of Compounds* **1**, **1a**, *and* **2**. At 125 MHz in C_5D_5N (**1**) or in $CDCl_3$ (**1a**, **2**); δ in ppm.

Position	**1**	**1a**	**2**
1	48.1 (*t*)	43.6 (*t*)	41.3 (*t*)
2	69.3 (*d*)	69.9 (*d*)	64.3 (*d*)
3	78.7 (*d*)	74.9 (*d*)	75.0 (*d*)
4	44.1 (*s*)	42.0 (*s*)	35.1 (*s*)
5	48.5 (*d*)	47.6 (*d*)	41.2 (*d*)
6	19.0 (*t*)	17.9 (*t*)	16.6 (*t*)
7	33.3 (*t*)[a])	31.9 (*t*)	31.4 (*t*)
8	40.2 (*s*)	39.4 (*s*)	38.6 (*s*)
9	48.6 (*d*)	47.7 (*d*)	46.5 (*d*)
10	38.9 (*s*)	37.9 (*s*)	37.1 (*s*)
11	24.2 (*t*)	23.5 (*t*)	22.0 (*t*)[b])
12	122.9 (*d*)	122.7 (*d*)	121.9 (*d*)
13	145.5 (*s*)	143.2 (*s*)	143.4 (*s*)
14	42.6 (*s*)	41.6 (*s*)	40.7 (*s*)
15	28.8 (*t*)	27.5 (*t*)	26.6 (*t*)
16	24.4 (*t*)	22.8 (*t*)	22.4 (*t*)[b])
17	47.5 (*s*)	46.6 (*s*)	45.8 (*s*)
18	41.8 (*d*)	40.0 (*d*)	39.2 (*d*)
19	41.7 (*t*)	40.1 (*t*)	39.1 (*t*)
20	37.0 (*s*)	34.4 (*s*)	34.8 (*s*)
21	29.5 (*t*)	28.5 (*t*)	27.3 (*t*)
22	33.1 (*t*)[a])	32.2 (*t*)	30.6 (*t*)
23	67.1 (*t*)	65.3 (*t*)	67.2 (*t*)
24	14.7 (*q*)	13.8 (*q*)	15.9 (*q*)[c])
25	17.8 (*q*)[d])	17.0 (*q*)[e])	16.0 (*q*)[c])
26	18.0 (*q*)[d])	16.9 (*q*)[e])	16.3 (*q*) [c])
27	26.6 (*q*)	25.7 (*q*)	25.0 (*q*)
28	180.6 (*s*)	182.9 (*s*)	182.0 (*s*)
29	74.3 (*t*)	74.5 (*t*)	73.3 (*t*)
30	20.2 (*q*)	19.2 (*q*)	18.0 (*q*)[f])
Me_2C	–	–	97.6 (*s*)
			18.2 (*q*)[f])
			28.3 (*q*)
2-AcO	–	170.3 (*s*), 20.7 (*q*)[g])	–
3-AcO	–	170.7 (*s*), 20.8 (*q*)[g])	–
23-AcO	–	170.4 (*s*), 20.8 (*q*)[g])	–
29-AcO	–	171.1 (*s*), 21.0 (*q*)[g])	–

[a])–[g]) Assignments may be interchanged.

a two-proton *singlet* when C(29) is oxygenated, whereas a well-defined *AB* system appears in the case of oxygenation of C(30) [4][5]. Therefore, we concluded that C(29) was hydroxylated in **1**. In the NOESY spectrum of the derivative **1a**, a strong NOE interaction was observed between Me(30) and Me(18), supporting this conclusion, C(29) occupying the α-equatorial position (*Fig. 1*).

The heavily overlapping signals at δ(H) 4.18–4.22 correlated with two oxygenated CH resonances at δ(C) 69.3 (*d*) and 78.7 (*d*), respectively, in the HMQC experiment of **1**. This indicated the presence of two secondary OH functions. To assign the position of

the two oxygenated methines, **1** was converted into its tetraacetate **1a**. In the ^{1}H-NMR spectra, the overlapping signals in **1** changed into two coupled signals at δ(H) 5.12 (*ddd,* J = 4.5, 4.2, 10.3 Hz, 1 H) and 5.04 (*d*, J = 10.3 Hz, 1 H) in **1a**. In the HMBC experiment (*Fig. 1*), the signal at δ(H) 5.12 correlated with δ(C) 74.9 (*d*, C(3)), 43.6 (*t*, C(1)), 42.0 (*s*, C(4)), 37.9 (*s*, C(10)), and 170.3 (*s*). The signal at δ(H) 5.04 correlated with δ(C) 69.9 (*d*, C(2)), 65.2 (*t*, C(23)), 42.0 (*s*, C(4)), 13.8 (*q*, C(24)), and 170.7 (*s*). These correlations suggested that the two secondary OH groups were located at C(2) and C(3). Analysis of NMR coupling constants indicated that the 2- and 3-OH groups were in α-equatorial and β-axial positions, respectively. Moreover, significant NOE correlations between H–C(2) and both Me(24) and Me(25), and between H–C(3) and H–C(5) further confirmed this conclusion (*Fig. 1*).

The two signals of **1** at δ(H) 3.72 (*d*, J = 7.0 Hz) and 4.18–4.20 (*m*, overlapped) correlated with the CH_2OH signal at δ(C) 67.1 (*t*) in an HMQC experiment, which indicated that either C(23) or C(24) was oxygenated. The diagnostic long-range correlations H–C(23)/C(24) (14.7 (*q*)), C(4) (44.1 (*s*)), C(5) (48.5 (*d*)), and C(3) (78.7 (*d*)) indicated that, indeed, C(23) was hydroxylated (*Fig. 1*). The NOE cross-peaks between Me(24) and H–C(2), H–C(23), and H–C(3) supported this.

Stachlic acid B (**2**) was obtained as an optically active, amorphous, colorless powder, with $[\alpha]_D^{18.1}$ = 34.4 (c = 0.6, $CHCl_3$). The molecular formula $C_{33}H_{52}O_6$ was established by HR-ESI-MS (*m*/*z* 543.3676 ($[M-1]^-$). The structure of **2** was established as (2α,3α)-2,29-dihydroxy-3,23-[(1,1-dimethylmethylene)dioxy]olean-12-en-28-oic acid by means of ^{1}H- and ^{13}C-NMR analyses (*Table*), in combination with HMQC, HMBC, and NOESY data (*Fig. 2*), and by comparison with the analytical data of **1**.

Compound **2** also gave a positive *Liebermann–Burchard* reaction typical for triterpenoids. The spectra of **2** were similar to those of **1**, which suggested that **2** also had an olean-12-en-28-oic acid skeleton (*Table*). The two-protons *singlet* at δ(H) 3.28 was assigned to the CH_2(29) group, in agreement with compound **1**. The ^{1}H-NMR spectrum of **2** showed signals of two oxygen-bearing CH at δ(H) 3.87 (*ddd*, J = 4.0, 3.0, 7.5 Hz, 1 H; δ(C) 64.3 (*d*)) and 3.76 (*d*, J = 3.0 Hz; δ(C) 75.0 (*d*)). The ^{1}H,^{1}H-COSY, HMQC, and HMBC data (*Fig. 2*) disclosed that the two O-bearing methines were located at C(2) and C(3), as in compound **1**. The two *doublet*s at δ(H) 3.66, 3.31 (2*d*, J = 12.0 Hz each, 1 H each) were assigned to CH_2(23) by HMQC and HMBC experiments (*Fig. 2*).

The difference between compounds **1** and **2** is the presence of two additional Me resonances at δ(C) 18.2 (*q*) and 28.3 (*q*), and of an additional quaternary C-atom at

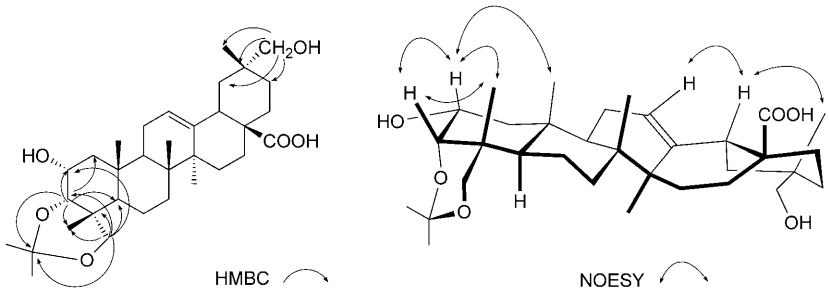


Fig. 2. *Key HMBC and NOESY correlations of* **2**

δ(C) 97.6 (*s*) in the ^{13}C-NMR spectrum. These data suggested an extra isopropylene (=1,1-dimethylmethylene) moiety in **2** [6]. The MS base peak at *m*/*z* 485 ([$M-1-C_3H_6O$]$^+$) further supported this conclusion. The HMBC long-range correlations between both H–C(3) and H–C(23) and the quaternary C-atom at δ(C) 97.6 (*s*) indicated that the isopropylene unit was attached at the two O-atoms at C(3) and C(23), as further substantiated by ^{13}C-NMR downfield shifts for C(3) and C(23).

The small coupling constant (J(2,3) = 3.0 Hz) suggested that the 3-O-atom was in an α-equatorial position, different from that in **1**. In the NOESY spectrum of **2**, cross-peaks for H–C(2)/H–C(3), H–C(2)/Me(24), H–C(2)/Me(25), and H–C(3)/Me(24) supported that the two oxygen-bearing groups at C(2) and C(3) were in α-equatorial positions. From these data, the structure of **2** was fully established. Note that compound **2** could be an artifact produced during the isolation procedure.

The purified triterpenoid **1** was found to have mild *in vitro* cytotoxic activity against human Hela cell lines, with an IC_{50} value of 18 μg/ml, as determined by classical MTT (=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) colorimetric assay (data not shown).

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Experimental Part

General. Commercial silica-gel plates (*Qing Dao Marine Chemical Group Co.*) were used for TLC analyses. M.p.: *XRC-1* micro-melting-point apparatus; uncorrected. UV/VIS Spectra: *Shimadzu UV-2401PC* spectrophotometer; λ_{max} in nm. Optical rotations: *Horiba SEAP-300* spectropolarimeter. IR Spectra: *Bio-Rad FTS-135* spectrophotometer; in cm^{-1}. ^{1}H- and ^{13}C-NMR as well as 2D-NMR spectra: *Bruker DRX-500* spectrometer; chemical shifts δ in ppm rel. to Me_4Si, coupling constant *J* in Hz. EI-MS *VG-Autospec-3000* mass spectrometer; in *m*/*z*.

Plant Material. The leaves and twigs of *Stachyurus himalaicus* var. *himalaicus* were collected in Wenshan County, Yunnan Province, P. R. China, in May 2003, and identified by Prof. *Zhi-Hao Hu*, Department of Botany, Yunnan University. A voucher specimen (No. 200305) was deposited at the Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education, Yunnan University.

Extraction and Isolation. The powdered plant material of *S. himalaicus* var. *himalaicus* (33 kg) was repeatedly extracted with EtOH at r.t. The extract was concentrated under reduced pressure to a brown syrup, which was partitioned between H_2O and petroleum ether (PE), AcOEt, and BuOH, successively. The AcOEt-soluble fraction (700 g) was subjected to column chromatography (CC) on silica gel (SiO_2), eluting with PE/AcOEt 20:1 → 1:1, AcOEt/MeOH 10:1 → 1:1, and MeOH to afford 19 fractions (*Fr. 1–19*). *Fr. 16* and *Fr. 11* were resubmitted to CC (*Pharmadex LH-20* and *RP C-18*) to yield **1** (50 mg) and **2** (8 mg), resp.

Acetylation of **1**. A mixture of **1** (10 mg), Ac_2O (2 ml), and pyridine (2 ml) was heated at 80° for 2 h. Ice-water was added, and the resulting precipitate was filtered to yield **1a** (9 mg) as an amorphous powder.

Stachlic Acid A (=*(2α,3β)-2,3,23,29-Tetrahydroxyolean-12-en-28-oic Acid*; **1**). Colorless needles. M.p. 287–289°. UV (MeOH): 204. $[\alpha]_D^{25}$ = 47.7 (*c* = 0.72, MeOH). IR (KBr): 3429, 2933, 2881, 1729, 1699, 1633, 1455, 1388, 1040, 1023, 1007. ^{1}H-NMR (500 MHz, (D_5)pyridine): 5.49 (*s*, H–C(12)); 4.18–4.22 (*m*, H_β–C(2), H_α–C(3), 1 H of CH_2(23)); 3.72 (*d*, *J* = 7.0, 1 H of CH_2(23)); 3.56 (*s*, CH_2(29)); 3.41 (*dd*, *J* = 4.4, 13.9, H–C(18)); 2.30 (*dd*, *J* = 2.9, 11.8, H_β–C(1)); 2.02–1.98 (*m*, H–

C(11)); 1.57–1.54 (*m*, CH_2(19)); 1.21 (*s*, Me(30)); 1.20 (*s*, Me(27)); 1.06 (*s*, Me(24), Me(25), Me(26)). ^{13}C-NMR: see *Table*. FAB-MS: 505 (100, $[M+1]^+$), 469 (46), 410 (30), 368 (25), 337 (42), 296 (20), 265 (15), 241 (5). HR-ESI-MS: 527.3341 ($[M+Na]^+$, $C_{30}H_{48}NaO_6^+$; calc. 527.3349).

(2α,3β)-2,3,23,29-Tetraacetoxyolean-12-en-28-oic Acid (**1a**). Colorless, amorphous powder. IR (KBr): 3437, 2925, 2854, 1744, 1638, 1244, 1043. ^{1}H-NMR (500 MHz, $CDCl_3$): 5.26 (*s*, H–C(12)); 5.12 (*ddd*, *J* = 4.5, 4.2, 10.3, H–C(2)); 5.04 (*d*, *J* = 10.3, H–C(3)); 3.81, 3.54 (2*d*, *J* = 11.8 each, CH_2(23)); 3.75, 3.69 (2*d*, *J* = 10.7 each, CH_2(29)); 2.83 (*dd*, *J* = 3.4, 13.3, H–C(18)); 2.06, 2.05, 1.99, 1.95 (4*s*, 4 AcO); 1.07 (*s*, Me(27)); 1.05 (*s*, Me(25)); 0.96 (*s*, Me(30)); 0.84 (*s*, Me(24)); 0.70 (*s*, Me(26)). ^{13}C-NMR: see *Table*. EI-MS: 672 (2, M^+), 568 (5), 306 (35), 288 (20), 259 (18), 246 (26), 233 (100), 201 (65), 187 (43). HR-ESI-MS: 695.3711 ($[M+Na]^+$, $C_{38}H_{56}NaO_{10}^+$; calc. 695.3759).

Stachlic acid B (= *(2α,3α)-2,29-Dihydroxy-3,23-[(1,1-dimethylmethylene)dioxy]olean-12-ene-28-oic Acid*; **2**). Colorless, amorphous powder. $[\alpha]_D^{18.1} = 34.4$ (*c* = 0.6, $CHCl_3$). IR (KBr): 3433, 2930, 2858, 1726, 1069, 1697. ^{1}H-NMR (500 MHz, $CDCl_3$): 5.32 (*s*, H–C(12)); 3.87 (*ddd*, *J* = 4.0, 3.0, 7.5, H–C(2)); 3.76 (*d*, *J* = 3.0, H–C(3)); 3.66, 3.31 (2*d*, *J* = 12.0 each, CH_2(23)); 3.28 (*s*, CH_2(29)); 2.87 (*dd*, *J* = 4.0, 13.5, H–C(18)); 1.42, 1.39 (*s*, Me_2C); 1.17 (*s*, Me(27)); 0.97 (*s*, Me(25)); 0.96 (*s*, Me(30)); 0.75 (*s*, Me(26)); 0.71 (*s*, Me(24)); ^{13}C-NMR: see *Table*. ESI-MS: 543 ($[M-1]^-$), 485 ($[M-1-C_3H_6O]^-$), 325, 279, 265, 221. HR-ESI-MS: 543.3676 ($[M-1]^-$, $C_{33}H_{51}O_6^-$; calc. 543.3686).

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